

# Three kinds of binding site for tentoxin on isolated chloroplast coupling factor 1

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**Abstract** Tentoxin binding on chloroplast coupling factor 1 (CF<sub>1</sub>) was studied using a centrifugation column method followed by HPLC analysis. From non-linear regression analysis of the results, the presence of three types of binding site with the following  $K_d$  values was deduced:  $6.9 \times 10^{-8}$  M (first site),  $1.4 \times 10^{-5}$  M (second site), and  $6.3 \times 10^{-3}$  M (third site). The binding of one tentoxin inhibits, that of two tentoxins moderately restores, and that of three tentoxins greatly stimulates the ATPase activity of CF<sub>1</sub>. The forward rate constant of the binding of tentoxin on the first site was  $6.3 \times 10^3$  M<sup>-1</sup> s<sup>-1</sup>.

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**Key words:** Chloroplast ATP synthase; Chloroplast coupling factor 1; F<sub>1</sub>-ATPase; Energy-transfer inhibitor; Tentoxin

## 1. Introduction

Tentoxin, a cyclic tetrapeptide (cyclo[L-leucyl-*N*-methyl-*trans*-dehydrophenylalanyl-glycyl-methyl-L-alanyl]) produced by the fungus *Alternaria alternata* causes species-specific chlorosis in sensitive higher plants [1]. One of its main targets is the CF<sub>1</sub> (chloroplast F<sub>1</sub>-ATPase) [2] where it acts as an energy-transfer inhibitor in photophosphorylation [3]. It was recently found that an F<sub>1</sub> from the cyanobacterium *Anacystis nidulans* is also sensitive to tentoxin [4].

F<sub>1</sub>, a hydrophilic portion of F<sub>0</sub>F<sub>1</sub>-ATP synthase, is composed of five different subunits,  $\alpha$ - $\epsilon$ , in order of decreasing molecular weight, with a stoichiometry of  $\alpha_3\beta_3\gamma\delta\epsilon$  [5–7]. The tentoxin binding sites on CF<sub>1</sub> seem to be located at least on the  $\beta$ -subunit, since *Rhodospirillum rubrum* chromatophore, itself insensitive to tentoxin, became sensitive after the  $\beta$ -less F<sub>1</sub> was reconstituted with the heterologous  $\beta$ -subunit of tentoxin-sensitive CF<sub>1</sub> from spinach [8]. Avni et al. [9] reported that tentoxin sensitivity was determined by codon 83 of the  $\beta$ -subunit in several species of the genus *Nicotiana*. When codon 83 was aspartic acid, it became sensitive to tentoxin, and when it was glutamic acid, it became resistant to tentoxin. Recently, Hu et al. [10] confirmed the importance of codon 83 by site-directed mutagenesis in *Chlamydomonas reinhardtii* CF<sub>1</sub>. Residue 83, however, is located in the  $\beta$ -barrel domain [11], far from nucleotide and Pi binding sites. No function has been assigned to it yet.

Tentoxin has the peculiar property of inhibiting ATPase activity in isolated sensitive CF<sub>1</sub> at low concentrations

(<10<sup>-6</sup> M) but stimulating it at high concentrations (>10<sup>-5</sup> M) [12]. Although it was reported that photophosphorylation inhibited by low concentrations of tentoxin did not recover in high concentrations of tentoxin [12], recent studies indicated that light-driven ATP synthesis was slightly restored at high concentrations of tentoxin [13]. These results indicate the existence of at least two types of tentoxin-binding sites on CF<sub>1</sub>. From the stimulating effects of tentoxin on ATPase, the  $K_d$  of the low-affinity site for tentoxin was estimated to be >1.6×10<sup>-4</sup> M [12]. Direct measurements of  $K_d$  values for tentoxin-binding sites have also been reported. For the first binding site (high-affinity site), Steele et al. [14] determined the  $K_d$  of lettuce CF<sub>1</sub> to be 5.5×10<sup>-9</sup> M by ultrafiltration. Dahse et al. [15] determined the  $K_d$  of spinach CF<sub>1</sub> to be 8.7×10<sup>-8</sup> M using HPLC techniques. Recently, Pinet et al. [16] extended these studies using isotopically labeled tentoxin, and found two types of binding site on spinach CF<sub>1</sub> at 4°C: 1.3 sites/CF<sub>1</sub> with  $K_{d1}$  = 1.2×10<sup>-6</sup> M and 1.5 sites/CF<sub>1</sub> with  $K_{d2}$  = 6.7×10<sup>-5</sup> M. Their  $K_{d1}$  value seems to be considerably larger than those of Steele et al. [14] and Dahse et al. [15], and than those estimated from the inhibition of ATPase activity of isolated CF<sub>1</sub> [12]. Their  $K_{d2}$  value seems to be rather small compared with those estimated from the activation of ATPase activity of isolated CF<sub>1</sub> [12].

As the  $\beta$ -subunit is involved in tentoxin binding and as each CF<sub>1</sub> has three  $\beta$ -subunits, the existence of three binding sites for tentoxin is plausible but has not been experimentally shown yet. If CF<sub>1</sub> has three tentoxin-binding sites, it would be interesting to see if the ATPase activity of C·T<sub>2</sub> (CF<sub>1</sub> which binds two molecules of tentoxin) and that of C·T<sub>3</sub> is the same or not. However, this has not been studied. As there are large differences among the reported  $K_d$  values (e.g. [14] vs. [16]) and the possible existence of a third type of binding site has not been carefully studied, we studied the binding of tentoxin on CF<sub>1</sub> using column centrifugation over tentoxin concentrations ranging from 10<sup>-8</sup> to 4×10<sup>-3</sup> M. Our results strongly suggest that CF<sub>1</sub> actually has three tentoxin-binding sites. This conclusion was reinforced by the results of tentoxin concentration on ATPase activity.

## 2. Materials and methods

### 2.1. Preparation of CF<sub>1</sub>

CF<sub>1</sub> was extracted from spinach chloroplasts by the chloroform method of Younis et al. [17], and purified in a buffer containing 10 mM Tricine-KOH (pH 8.0), 1 mM EDTA, and 2 mM ATP by Sepharose CL-4B and HiPrep Sephacryl S-300 (Pharmacia) columns, followed by sucrose density-gradient (15–35%, w/v) ultracentrifugation (140 000×g, 22 h). The purified CF<sub>1</sub> was precipitated by 50% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and stored at 4°C. The CF<sub>1</sub> preparation contained substoichiometric amounts of  $\delta$ -subunits on SDS-PAGE analysis as reported by McCarty and Moroney [5] (data not shown). Before use, CF<sub>1</sub> precipitate was dissolved in 1 mM Tricine-KOH

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**Abbreviations:** CF<sub>1</sub>, chloroplast coupling factor 1; C·T<sub>*n*</sub>, CF<sub>1</sub> which binds *n* molecules of tentoxin;  $K_d$ , dissociation constant; PCA, perchloric acid

(pH 8.0) unless otherwise indicated, and the solution was passed slowly through a Sephadex G-50 (1.0 cm $\times$ 20 cm) column equilibrated with 1 mM Tricine-KOH (pH 8.0) at room temperature. It took about 45 min before CF<sub>1</sub> was eluted from the column. The concentration of CF<sub>1</sub> in the effluent from a typical column was determined by the absorbance at 278 nm assuming  $A_{278\text{nm}}=0.53$  for CF<sub>1</sub> solution at 1 mg/ml (Hisabori and Sakurai, unpublished). Trypsin-activated CF<sub>1</sub> was obtained by incubation with 0.1 mg/ml trypsin in 2 mM ATP, 1 mM EDTA, and 30 mM Tricine-NaOH (pH 8.0) at room temperature for 5 min. The activation was stopped by adding soybean trypsin inhibitor to 0.25 mg/ml.

## 2.2. Tentoxin binding on CF<sub>1</sub>

Solutions of native or trypsin-activated CF<sub>1</sub> were incubated at room temperature with various concentrations of tentoxin (either purchased from Sigma Chemical Co., St. Louis, MO, or kindly donated by Mr. T. Fukushima of JT Co., Yokohama) for the time as indicated. For the determination of tentoxin bound on CF<sub>1</sub>, a portion of this mixture, containing CF<sub>1</sub>, was centrifuged through a Sephadex G-50 column [18] to remove free tentoxin. The effluent was treated with PCA to 2.6% (w/v), and neutralized with 0.35 M KCl, 0.18 M Tricine-KOH (pH 9.0), and 0.18 mM EDTA, and centrifuged. Tentoxin in the neutralized supernatant was separated on a reverse phase HPLC column (Merck, LiChrospher 100 RP-18(e), 4.6 mm $\times$ 25 cm), with 40% (v/v) acetonitrile-0.06% (v/v) trifluoroacetic acid as the elution buffer (0.5 ml/min) and analyzed at 285 nm with UV detector (SPD-6A, Shimadzu). Recovery of CF<sub>1</sub> from the centrifugation column was estimated using the protein determination method of Bradford [19] (>0.2 mg/ml CF<sub>1</sub>) or from the recovery of CF<sub>1</sub>-bound ADP (bound ADP/CF<sub>1</sub>=1.13) after column centrifugation. The effluent was treated with PCA and neutralized with 175 mM K<sub>2</sub>CO<sub>3</sub>. ADP was determined as ATP by luciferase-luciferin assay after incubation with pyruvate kinase and phosphoenolpyruvate.

## 2.3. Effects of tentoxin on ATPase activity of CF<sub>1</sub>

For concentrations of tentoxin less than  $1.0 \times 10^{-6}$  M, solutions of native CF<sub>1</sub> ( $2.3 \times 10^{-9}$  M) were pre-incubated with various concentrations of tentoxin, 1.3 mM ATP, 1.3 mM EDTA and 0.7 mM Tricine-KOH (pH 8.0) for 60 min at room temperature. The ATPase assay (at 37°C) was initiated by adding the assay solution containing the same concentrations of tentoxin as in the pre-incubation mixture, and MgCl<sub>2</sub> to a final concentration of 1.4 mM. The reaction was terminated by injecting a portion of the reaction mixture into an HPLC column (Shodex AXPak WA-624, 6.0 mm $\times$ 15 cm, Showadenko), and the amount of ATP hydrolyzed was determined from ADP at 259 nm essentially according to the HPLC method of Pinet et al. [20]. For concentrations of tentoxin greater than  $1.0 \times 10^{-6}$  M, solutions of CF<sub>1</sub> ( $2.3 \times 10^{-7}$  M) were incubated as above, and the ATPase activity was determined as above except that the concentration of ATP was 4 mM, and the reaction times ranged from 2 to 15 min. The reaction was stopped by adding trichloroacetic acid to 2.7% (w/v). Pi liberated in the reaction mixture was determined by colorimetry after reducing the phosphomolybdate with SnCl<sub>2</sub>, according to the methodology developed for the Autoanalyzer of Technicon Co. (1966).

Trypsin-activated CF<sub>1</sub> ( $1.27 \times 10^{-7}$  M) was incubated with  $5.0 \times 10^{-7}$  M tentoxin at room temperature for the times indicated. ATPase activity was then assayed at 37°C in a reaction mixture containing 1 mM ATP, 0.75 mM MgCl<sub>2</sub>, and 15 mM Tricine-NaOH (pH 8.0). After 3 min, the reaction was stopped by adding trichloroacetic acid to 2.7% (w/v), and Pi in the acidified reaction mixture was determined as described above.

## 3. Results and discussion

Fig. 1 shows the binding of tentoxin to CF<sub>1</sub> studied over a range of tentoxin concentrations from  $10^{-8}$  M to  $4 \times 10^{-3}$  M, the latter being the solubility limit of tentoxin in water [20]. The binding curve clearly indicates several types of binding site. We analyzed the results, assuming two or three types of saturable (Michaelis-Menten type) binding site on CF<sub>1</sub>, with a non-linear regression procedure using the computer program 'GraFit' (Erithacus Software Ltd., Staines). Assuming three

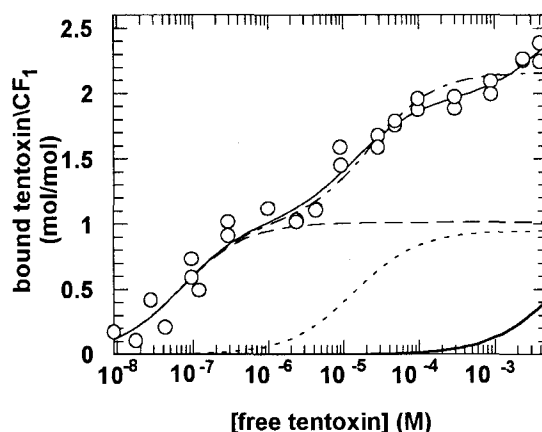


Fig. 1. Relation between free tentoxin concentration and the amount of tentoxin bound on native CF<sub>1</sub>. Binding of tentoxin (initial concentration:  $10^{-9}$ – $4 \times 10^{-3}$  M) by concentrations of CF<sub>1</sub> ranging from  $5.8 \times 10^{-9}$  M to  $1.2 \times 10^{-6}$  M incubated for 60 min was measured as described in Section 2. Free tentoxin concentration was calculated by subtracting the bound tentoxin from the total added.  $\circ$ , Experimentally obtained values. Theoretical curves assuming three kinds of binding site with different affinities: (---), deduced binding to first binding site; (- - -), deduced binding to second binding site; (bold solid line), deduced binding to third binding site; (solid line): the best-fit curve (sum of the total tentoxin bounds). Theoretical curve (— · —) assuming two kinds of binding site with different affinities. For details, see text.

types of binding sites, we obtained deduced kinetic values ( $\chi^2 = 1.20 \times 10^{-2}$ ) of  $1.01 \pm 0.10$ ,  $0.95 \pm 0.11$ , and  $0.95 \pm 1.95$  binding sites/CF<sub>1</sub>, with  $K_d$  values of  $6.92 \times 10^{-8}$  M (first site,  $K_{d1}$ ),  $1.35 \times 10^{-5}$  M (second site,  $K_{d2}$ ), and  $6.31 \times 10^{-3}$  M (third site,  $K_{d3}$ ), respectively. The number of the third type of binding site per CF<sub>1</sub> and its  $K_d$  value were difficult to determine very accurately because only about 0.4 mol of tentoxin binds to this third site even at the solubility limit of tentoxin. Assuming two types of binding site, we obtained deduced kinetic values ( $\chi^2 = 1.58 \times 10^{-2}$ ) of 1.01 and 1.15 binding sites/CF<sub>1</sub> with  $K_d$  values of  $6.92 \times 10^{-8}$  M and  $2.23 \times 10^{-5}$  M, respectively. Holding the kinetic values for the first site steady at 1.01 site/CF<sub>1</sub> with a  $K_d$  value of  $6.92 \times 10^{-8}$  M, we then subtracted the deduced tentoxin binding on the first site from the experimental value to obtain binding value(s) on the other binding site(s). Analysis of the results at a concentration range of  $2 \times 10^{-6}$ – $4 \times 10^{-3}$  M tentoxin gave  $\chi^2$  values of  $9.29 \times 10^{-3}$  and  $1.52 \times 10^{-2}$ , respectively, for the three- and two-site assumptions. Comparison of these  $\chi^2$  values shows that the three-site assumption gives a better fit than the two-site assumption. This is also suggested by a comparison of the two theoretical binding curves with the data obtained experimentally at  $>10^{-4}$  M tentoxin (Fig. 1).

The effects of tentoxin concentration on the ATPase activity of native CF<sub>1</sub> are shown in Fig. 2A. At  $<10^{-6}$  M tentoxin, activity was inhibited, and at  $>10^{-6}$  M, it was recovered or stimulated as reported previously [12]. The activity was not saturated by concentrations of tentoxin higher than  $10^{-3}$  M, consistent with the results of Steele et al. [12]. The tentoxin concentration required for 50% of maximum activity was clearly higher than  $10^{-3}$  M. Using  $K_d$  values deduced from the three-site model in Fig. 1, we calculated the ratio of four states of CF<sub>1</sub> with respect to number of tentoxin bound as a function of free tentoxin concentration: C·T<sub>0</sub>, C·T<sub>1</sub>, C·T<sub>2</sub>, and

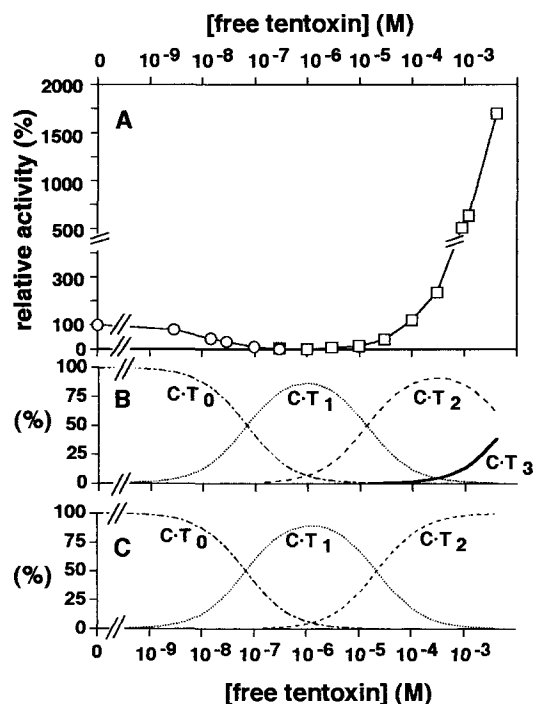
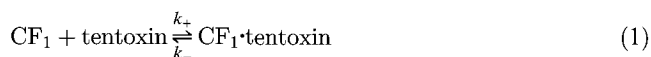


Fig. 2. A: Effects of tentoxin on ATPase activity of native CF<sub>1</sub>. ○: The concentration of CF<sub>1</sub> was 2.3 × 10<sup>-9</sup> M and ATPase activity was determined from the product ADP by the HPLC method. □: The concentration of CF<sub>1</sub> was 2.3 × 10<sup>-7</sup> M and ATPase activity was determined by colorimetry of Pi. ATPase activity of CF<sub>1</sub> in the absence of tentoxin was 0.29 μmol/mg/min. Free tentoxin concentration was calculated from the kinetic values obtained in Fig. 1, assuming three binding sites. B: Relative proportions of C-T<sub>n</sub> deduced by assuming three tentoxin-binding sites. C: Relative proportions of C-T<sub>n</sub> deduced by assuming two tentoxin-binding sites.

C-T<sub>3</sub> (Fig. 2B). Our data, at concentrations of tentoxin less than 10<sup>-6</sup> M, showed the activity of C-T<sub>1</sub> to be almost zero. At 10<sup>-5</sup> M tentoxin, we calculated that about 60% of CF<sub>1</sub> was C-T<sub>1</sub>, and about 40% C-T<sub>2</sub>. At this concentration, the total activity was less than 40% that of free CF<sub>1</sub> (C-T<sub>0</sub>), indicating that C-T<sub>2</sub> was less active than C-T<sub>0</sub>. At 4 × 10<sup>-3</sup> M tentoxin, about 60% of CF<sub>1</sub> was C-T<sub>2</sub>, and about 40% C-T<sub>3</sub>. The activity was then about 1700% of C-T<sub>0</sub>. If we assume that the activity of C-T<sub>2</sub> is 50% that of C-T<sub>0</sub>, the activity of C-T<sub>3</sub> would be about 4000% that of C-T<sub>0</sub>. If we assume that CF<sub>1</sub> has only two kinds of binding site (Fig. 2C), the deduced  $K_{d2}$  is 2.2 × 10<sup>-5</sup> M (Fig. 1). This is very different from the tentoxin concentration required to stimulate ATPase activity to half maximum, as shown in Fig. 2A (>10<sup>-3</sup> M). The results shown in Fig. 2 clearly favor the three-site model. The value of  $K_{d1}$  was estimated to be in the order of 10<sup>-8</sup> M (Fig. 2A), not very different from values deduced from the inhibition of ATPase (1 × 10<sup>-8</sup> M [12], <10<sup>-8</sup> M [15], 3 × 10<sup>-7</sup> M [21]).

Fig. 3 shows the time course of the binding of a low concentration of tentoxin (5.0 × 10<sup>-7</sup> M) to native or trypsin-treated CF<sub>1</sub>. We analyzed the results on the basis of Equation 1 using the computer program 'KaleidaGraph' (Synergy Software, Reading), neglecting the binding on the second and third sites at this low concentration of tentoxin:



where  $k_- = K_{d1} \times k_+$  ( $K_{d1}$  being 6.9 × 10<sup>-8</sup> M). We thus ob-

tained values of  $k_+ = 6.3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_- = 4.3 \times 10^{-4} \text{ s}^{-1}$ . This  $k_-$  value indicates that less than 1% of tentoxin bound on the first site dissociates from CF<sub>1</sub> in 20 s. This slow dissociation assures that the column centrifugation method is suitable for analyzing tentoxin binding at least on the first site. Trypsin treatment greatly activates ATPase activity [22], but did not significantly affect the binding of tentoxin on the first site of CF<sub>1</sub> (Fig. 3).

We also estimated the value of  $k_+$  by analyzing the time course of inhibition of ATPase on the basis of Eq. 1 and  $K_{d1} = 6.9 \times 10^{-8} \text{ M}$ , assuming that C-T<sub>1</sub> has no activity (Fig. 4). For this purpose, we used trypsin-activated CF<sub>1</sub>, because it has much higher activity than native CF<sub>1</sub>, while its tentoxin-binding properties are similar (Fig. 3). We thus obtained values of  $k_+ = 6.9 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_- = 4.8 \times 10^{-4} \text{ s}^{-1}$ . These values agreed well with those obtained from the direct binding assay (Fig. 3), suggesting the accuracy of our estimated  $k$  values.

The value of  $K_{d1}$ , estimated from the results shown in Fig. 1, was 6.9 × 10<sup>-8</sup> M, not very different from some previously reported values (5 × 10<sup>-9</sup> M [14], 8.7 × 10<sup>-8</sup> M [15]), though Pinet et al. [16] reported the much larger value of 1.2 × 10<sup>-6</sup> M. Our CF<sub>1</sub> preparation contained substoichiometric amounts of a δ-subunit. Recent results suggest that this δ-subunit touches the β-barrel domain of the β-subunit [23,24], which binds tentoxin. However, a low δ content did not seem to affect the  $K_{d1}$  value of our CF<sub>1</sub> significantly. This is suggested by the following: (1) the value of  $K_{d1}$  of a five-subunit CF<sub>1</sub> (8.7 × 10<sup>-8</sup> M [15]) was similar to ours; and (2) competition experiments for tentoxin binding between a five-subunit CF<sub>1</sub> and a CF<sub>1</sub> lacking the δ- and ε-subunits, as estimated by inhibition of ATPase activity by tentoxin in the latter, indicated that they did not much differ from each other in their binding affinities (affinities of the CF<sub>1</sub> lacking the δ- and ε-subunits for tentoxin were higher by a factor of less than 3, as shown in Fig. 2 of [25]). Our results shown in Fig. 3 indicate that the trypsin treatment of CF<sub>1</sub>, which would eliminate the δ- and ε-subunits and partially digest the γ-subunit, did not significantly affect the affinity of CF<sub>1</sub> for tentoxin. The cause for the much larger  $K_{d1}$  value reported by Pinet et al. [16] is not clear, but it might be due to the low temperature (4°C) which they chose for incubation.

We have shown that CF<sub>1</sub> has three kinds of binding site for tentoxin, with different affinities, and that binding of tentoxin

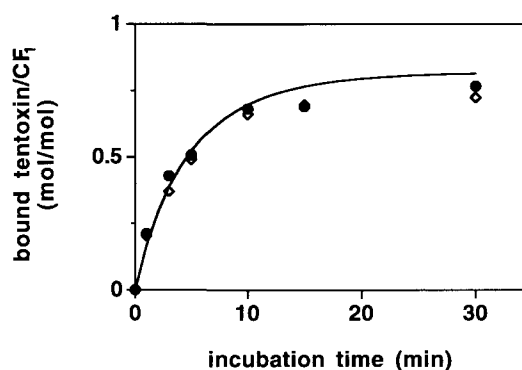


Fig. 3. Time course of the binding of a low concentration of tentoxin to native or trypsin-treated CF<sub>1</sub>. Initial concentrations of CF<sub>1</sub> and tentoxin were 2.3 × 10<sup>-7</sup> M and 5.0 × 10<sup>-7</sup> M, respectively. Experimentally obtained value: ●, native CF<sub>1</sub>; ◇, trypsin-treated CF<sub>1</sub>; line: the deduced theoretical curve (see text).

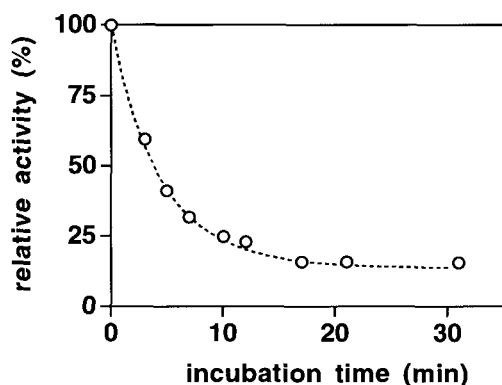


Fig. 4. Time course of inhibition of ATPase activity of trypsin-treated CF<sub>1</sub> by a low concentration of tentoxin.  $1.27 \times 10^{-7}$  M of trypsin-treated CF<sub>1</sub> and  $5.0 \times 10^{-7}$  M tentoxin were mixed at time zero, at room temperature. A portion of the mixture was then transferred to an assay mixture at specified times, and ATPase activity was assayed for 3 min. Except for the zero time control which was assayed in the absence of tentoxin (activity:  $7.5 \mu\text{mol/mg/min}$ ), the time indicated was 1.5 min after the onset of each assay. ○: Experimentally obtained value; (---): the deduced theoretical curve (see text).

on these sites differentially affects the ATPase activity of CF<sub>1</sub>. This three-site model finds its counterpart in nucleotide binding on CF<sub>1</sub>, as revealed by structural studies of mitochondrial F<sub>1</sub> (pseudo-threefold symmetry) [11], and by functional studies of CF<sub>1</sub> [5,26]. The nucleotide binding sites not only have different affinities, but the binding of nucleotides also differentially affects ATPase kinetics in a complex manner. The relationship between the  $\beta$ -subunits concerned in the high-affinity binding of tentoxin and in tight ADP binding remains to be investigated.

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